Original

Evaluation of bioactive glass and platelet-rich plasma for bone healing in rabbit calvarial defects

Luiz A. M. Penteado^{1,2}), Carlos E. D. Colombo³), Roberta A. P. M. Penteado²),
Angélica O. Assis⁴), and Bruno C. V. Gurgel⁴)

¹)Department of Dentistry, Federal University of Alagoas, Maceió, AL, Brazil

²)Department of Dentistry, University Center of Maceió, Maceió, AL, Brazil

³)Department of Dentistry, Taubaté University, São Paulo, SP, Brazil

⁴)Department of Dentistry, Federal University of Rio Grande do Norte, Natal, RN, Brazil

(Received November 5, 2012; Accepted July 5, 2013)

Abstract: Bone regeneration is an important objective in clinical dental practice and has been used for different applications. The aim of this study was to evaluate the effectiveness of platelet-rich plasma (PRP) and bioactive glass (BG) for bone healing of surgical calvarial defects in rabbits. Two 8-mm defects were prepared in the parietal bones of ten animals, and the animals were randomly assigned to two groups. In each group, two subgroups were created with five defects each: BC - blood clot, BG, PRP and PRP + BG. Thus, four treatments were performed with five specimens each. The animals were sacrificed after 12 weeks and the specimens were analyzed radiographically, histologically and histomorphometrically. Data were subjected to ANOVA and Tukey's tests ($\alpha = 0.05$). Outcomes demonstrated that the PRP group had higher bone density (%) values than the groups not treated with PRP ($P < 0.05$). Histometrically, both groups treated with PRP (PRP: 25.6 ± 9.9 ; PRP+BG: 25.8 ± 12.4) demonstrated higher percentages of new bone formation than the groups not treated with PRP (BG: 6.1 ± 4.3 ; BC: 7.8 ± 5.6) ($P < 0.05$). The results suggested that PRP improved bone repair and that bioactive glass alone, or in association with PRP, did not improve bone healing.

(J Oral Sci 55, 225-232, 2013)

Keywords: bone regeneration; healing; bioactive glass; platelet-rich plasma; rabbit.

Introduction

Bone regeneration is a desirable objective in dental clinical practice. Regeneration of bone can be used for correction of traumatic bone loss or atrophic changes in the alveolar processes, or even for bone reconstruction after bone loss due to progression of periodontal disease. Several studies, in both animals and humans, have reported that bone regeneration can be achieved by bone grafting, with or without the use of growth factors (1-5).

Growth factors have an important role in the regulation and stimulation of wound healing, affecting cellular processes such as mitogenesis, chemotaxis, differentiation and metabolism (5-7). Platelets contain many of these growth factors (platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- β), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF-I) and epithelial growth factor (EGF)) and platelet-rich plasma (PRP) can be obtained in high concentrations by centrifugation. The application of PRP was initially proposed by Marx et al. (6) and Whitman et al. (8) to enhance wound healing. Currently, PRP is used during implant placement, for the treatment of peri-implant bone defects, and for sinus-lift procedures, as well as for maxillofacial and periodontal reconstructive surgery (9,10).

When used alone, PRP does not seem to exert any additional effects when compared with blood clots (11-14). For this reason, PRP has been used in association

Correspondence to Dr. Bruno Cesar de Vasconcelos Gurgel, Federal University of Rio Grande do Norte – UFRN, Senador Salgado Filho Ave, 1787, Lagoa Nova, 59056-000, Natal, RN, Brazil

Fax: +55-84-3215-4101 E-mail: bcgurgel@yahoo.com.br

with bone grafts, as it can act as a scaffold material, since a high concentration of platelets can increase the local concentration of secreted growth factors inside bone defects and enhance bone regeneration. Marx et al. (6) demonstrated that a combination of bone graft substitutes and growth factors such as cytokines contained in PRP might be suitable for enhancing bone healing and density.

Basic studies have shown that application of PRP enhances early bone formation (12,15-17) or might have additional beneficial effects on bone regeneration (18-23), when used alone or mixed with bone grafts (4,14,24-26). However, the use of highly concentrated PRP has also been considered prejudicial to the repair of non-critical defects in the rabbit calvaria (27). Nagata et al. (19) have also indicated that there might be an optimal concentration of PRP beyond which harmful effects may occur.

Autogenous bone grafts are the gold standard for regeneration of bony defects in the craniofacial region. However, a variety of osteoconductive bone substitutes have been reported. With regard to clinical applications, other studies have used PRP in association with some bone substitutes, such as chitosan (28), particulate bone grafts (29-31), anorganic bovine bone allografts (1,3,17) and bioactive glass (32). Bioactive glass (BG) is a type of bioactive ceramic that has also been used in some studies to enhance bone formation (4,32-35). Demir et al. (4) tested a combination of BG and platelet-rich plasma for use in bone defects, and suggested that both the PRP/BG combination and BG alone were effective. Keles et al. (34) also demonstrated a significant effect when bone defects were filled with PRP and BG. These studies demonstrated that PRP and BG were effective for reduction of pocket depth, filling of defects, and enhancement of clinical attachment gain.

Our hypothesis in designing the present study was that healing of a bone defect filled with BG would be more rapid when PRP was added. Accordingly, using radiographic, histologic and histomorphometric techniques, we evaluated the effects of BG and PRP for healing of calvarial defects in rabbits.

Materials and Methods

Animals

Ten male adult New Zealand rabbits (3.5 kg) were employed in the study. The animals were kept in plastic cages with access to food and water *ad libitum*. Prior to surgical creation of the calvarial defects, all animals were allowed to acclimate to the laboratory environment for a period of 7 days. The protocol was approved by the institutional Ethics Committee (027A/2006).

Surgical procedure

Twenty-four hours before the surgical procedures, the animals were fasted. General anesthesia was induced by intramuscular administration of 65 mg/kg ketamine and 4 mg/kg xylazine (36). Anesthesia was monitored and maintained by a veterinarian during all surgical processes. After removal of hair over the dorsal cranium, the surgical site was scrubbed with 70% alcohol followed by 10% iodine, and a mid-sagittal linear incision was made through the skin of the scalp from the nasal bone to the mid-sagittal cristae. A full-thickness flap including the periosteum was reflected, exposing the calvarial bone (parietal and frontal bones). After dissection and bone exposure, two standardized circular defects, each 8 mm in diameter, were created on two sides of the parietal bones using a trephine drill, with a 170 x g velocity, 45N torque, and abundant irrigation with sterile saline solution, exposing the dura mater.

The animals were then randomly assigned to two groups to avoid any inter-group crossover effect: Group 1 – blood clot only (BC) and BG only, Perioglas (Nova-Bone Products, Alachua, FL, USA); Group 2 – PRP only and PRP + BG in combination (PRP + BG). Four treatments were thus performed with five specimens each.

PRP preparation

Before the surgical procedure, the animals were subjected to blood collection and hemogram analysis to determine the platelet count. Blood was obtained from all animals several minutes after induction of anesthesia, including those in the non-PRP group, and the same procedures were conducted. Blood was collected for hemogram analysis by puncture of the auricular superficial vein and cardiac puncture for PRP preparation.

For PRP preparation, blood was drawn from each rabbit into two 5-mL silicone tubes containing 0.5 mL of 3.2% sodium citrate solution as an anticoagulant. The tubes were centrifuged at 170c for 10 min at room temperature, and the blood was thus separated into three basic fractions: red blood cells (at the bottom of the tube), PRP (a discrete gray layer in the middle of the tube) and platelet-poor plasma (PPP; at the top of the tube). The portion corresponding to the platelet plasma was transferred by pipette from the tube to another empty tube. PPP was centrifuged again at 170 x g for 10 min, and approximately 50% of the supernatant, corresponding to the PPP, was removed; the platelet concentrate remaining was placed in a sterile vat at 37°C. Approximately 20 µL of 10% calcium chloride was then added to the preparation to activate the platelets and to form a platelet gel, after 10 to 15 min. When BG was

used, the graft was added immediately after addition of 10% calcium chloride. After formation of the gel, it was applied to predetermined bone defects, either alone or in combination with the BG.

After the bone defects had been filled, the soft tissues and periosteum were repositioned for total coverage and sewn with 4.0 resorbable suture (Vicryl suture 4.0, Ethicon Inc. /Johnson & Johnson, São José dos Campos, Brazil). After surgery, 1 mg/kg Flunixin Meglumine (Banamine, Schering-Plough Veterinary, Rio de Janeiro, Brazil) was administered by subcutaneous injection on three consecutive days, as well as antibiotics (Flotril 10% injection, Schering-Plough Veterinary) on the first and fourth days after surgery.

Histology and histomorphometry

After 3 months of healing, the animals were killed under general anesthesia, as described previously, followed by intravascular injection of 10 mL of 0.19% potassium chloride. The calvaria was removed and immediately fixed in buffered 10% formaldehyde solution (Lillie, pH 7.2), together with 100 mL of pure formaldehyde, 900 mL of deionized water, 6.5 g of Na_2HPO_4 and 4.0 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, for a period of 48 h.

Radiographic analysis

Radiographs (Kodak Ultra-speed) of each specimen were taken with a Gnatus Timex 70 dispositive (70 kVp, 7 mA), with a time exposure of 0.32 s and a standard object-focus distance. The radiographs were developed in an automatic developer (Air Techniques, A/T 2000 XR) and digitalized with an HP Scanjet 3670 scanner (600 dpi, 8 bit gray scale). The images were saved and the gray density was further analyzed using Image J Software (version 1.37, <http://rsb.info.nih.gov/ij>).

Radiographic density, using a gray scale, was obtained as the mean of three measurements performed for each bone defect, with a standardized cursor delimiting the entire defect. These measurements were performed by a blinded and previously calibrated evaluator ($R^2 = 0.9488$).

Preparation of histological sections

After fixing, the specimens were demineralized for 5 days in a mixture of two solutions: equal parts of 88% formic acid in distilled water and 20% sodium citrate in distilled water. For paraffin embedding, each defect was separated into two halves and included in the same block. Semi-serial paraffin sections (6 μm) were obtained from the middle to the margin of the defect, in an anterior-posterior direction, at six levels within a distance of 100

μm , totaling twelve levels for each block. From each level, two glass microscope slides containing two or three sections were prepared. Histological sections were stained with hematoxylin and eosin.

Histologic and histomorphometric evaluation

Histological sections were uniformly selected from the beginning to the end of the defect and analyzed by light microscopy at $\times 10$ magnification. Bone defects were evaluated histologically for linear closure of the bone defect, new bone trabeculae and blood vessel formation, bone tissue cells, bone marrow and possible inflammatory response.

Two histologic sections from each of the twelve levels obtained from the defect were subjected to histomorphometric analysis. New bone formation within the region bounded by the reversal lines in the area of the defect was measured using a light microscope at $\times 100$ magnification and an image analysis system (Image J). The percentage area of new bone corresponded to the mean value for the 12 levels analyzed in each defect for each treatment.

Statistical analysis

Means and standard deviations of radiographic bone density (%) and histomorphometric percentages of new bone formation were calculated for each group. Comparisons between groups were subjected to one-way analysis of variance (ANOVA) and Tukey's test (Bio Estat 4.0) at a significance level of 5% ($\alpha = 0.05$) and a 95% confidence interval.

Results

Radiographic analysis

Data from radiographic analyses are shown in Table 1. The outcomes demonstrated that the groups treated with PRP had higher bone density values than the groups not treated with PRP, and that the differences between the groups were statistically significant (Fig. 1).

Histological analysis

PRP + BG association

Histological evaluation of the defects revealed a central region rich in dense connective tissue, particles of BG and a large number of macrophages, mainly associated with the glass particles. At the edge of the defects, intense de novo bone formation activity was observed, and areas of new bone had already formed in the middle of the defect (Fig. 2).

PRP

Histological sections from the central region of the defect

Table 1 Mean percentages and standard deviations (SD) of bone density in defects

Treatment	Mean ± SD	Homogenous groups	F	P
PRP+BG	66.2 ± 4.3 A	A	14.9322	0.0003
PRP	60.7 ± 3.5 A	A		
BG	51.3 ± 4.9 B	B		
BC	47.1 ± 5.4 B	B		

Means followed by the same letter did not differ significantly in ANOVA and Tukey’s test ($P < 0.05$) CI = 95%.

Fig. 1 Radiographic images of parietal bones and bone defects in the respective groups. Bone densities were calculated for all groups. (I) Bioactive glass; (II) Blood clot; (III) PRP + BG; (IV) PRP. Groups treated with PRP (III and IV) showed significantly higher bone density than the groups not treated with PRP (I and II).

Table 2 Mean percentages and standard deviations (SD) for new bone formation according to treatments

Treatment	Mean ± SD	Homogenous groups	F	P
PRP+BG	25.6 ± 9.9	A	7.6673	0.0032
PRP	25.8 ± 12.4	A		
BG	6.1 ± 4.3	B		
BC	7.8 ± 5.6	B		

Means followed by the same letter did not differ significantly in ANOVA and Tukey’s test ($P < 0.05$) CI = 95%.

showed dense connective tissue and several foci of new bone formation within the area of the defect. However, the new bone was thin and did not fill the entire defect.

Bioactive glass

Initial histological sections were completely filled with dense connective tissue; there were many glass particles and macrophages, mainly around the biomaterial. Intense activity and areas of new bone formation were only observed at the edge of the defect, while in the middle of the defect there was evidence of connective tissue associated with bioactive glass and macrophages, but without bone formation (Fig. 3).

Blood clot

Initial histological sections from bone defects showed

Fig. 2 Histological aspects of the PRP + BG group. (A) Central region rich in dense connective tissue and particles of BG PRP+BG. Magnification ×40; (B) Middle of the defect with new bone formation and dense connective tissue. Magnification ×100; (C) Particle of bioactive glass with macrophages (arrow). Magnification ×400; (D) Edge of the defect and presence of osteoblasts (arrow). Magnification ×400.

Fig. 3 Histological evaluation of the BG group. (A) Histological aspect of the middle of the defect treated with BG showing a large quantity of glass particles as well as evidence of connective tissue; (B) Edge of the defect showing new bone formation. Magnification ×100.

Fig. 4 Histological aspects of the blood clot group at the edge of the defect. (A and B) Discrete new bone formation is evident only at the edge, and dense connective tissue fills the middle of the defect. Magnification ×10.

complete filling by dense connective tissue without new bone formation in the middle of the defects. Discrete new bone formation was observed only at the edges. However, new bone formation was observed in deep areas of the defects, while the central portion was still filled with dense connective tissue (Fig. 4).

Histomorphometric analysis

The data obtained by histomorphometric analyses

showed significant differences in the percentage of new bone formation among the groups ($P = 0.0032$). Both of the PRP groups demonstrated higher values of new bone formation than the groups not treated with PRP. On the other hand, no differences were observed between the groups with and without PRP treatment ($P > 0.05$) (Table 2).

Discussion

Several regenerative procedures have been proposed for evaluation of bone formation in bone defects. The use of bone substitutes, membranes and growth factors, together or separately, is the most common approach used to achieve bone regeneration. The objective of the present study was to clarify whether addition of platelet-rich plasma (PRP) and/or bioactive glass (BG) would have a positive effect on bone formation in rabbit calvarial defects. The results of radiographic, histological and histomorphometric examinations showed that PRP enhanced bone repair, either alone or in combination with BG.

PRP is an autologous source of several growth factors (PDGF, TGF- β , IGF) that have an important role in wound healing, promoting initial coagulation (37) and exerting strong angiogenic, mitogenic and capillary growth effects (38). As such, PRP has become a focus of interest because of its potential to accelerate cell proliferation, initiate mineralization *in vitro* (39), and enhance bone regeneration in humans (40). Kanno et al. (41) reported that PRP promoted the expression of an osteoblast-specific transcription factor (*cbfa-1*) that regulates the transcription of osteopontin, bone sialoprotein, osteoprotegerin and osteocalcin, which are essential for osteoblast differentiation (42,43).

Studies of the application of PRP to bone regeneration in oral surgery have yielded conflicting results, suggesting that factors such as the surgical site, type of bone used, diffusion of PRP and/or growth factors, as well as the concentration of blood platelets, may modulate responses (6,9,10,44,45). According to Mooren et al. (44), the critical effective amount of platelets for each type of animal, as well standardization of the platelet concentration in PRP, should be defined by experimental studies. Mooren et al. (45) also suggested that the difference between the metabolisms of small and large animals may also play a role in the rate of early bone healing.

The results of the present study are in agreement with the results of previous ones demonstrating that PRP significantly enhances bone formation in rabbit calvarial defects (17,19,28,31). After 3 months of implantation, the present study confirmed that no full defect closures

were evident in controls treated with a blood clot alone, as described previously (46). In addition, Aghaloo et al. (15) did not find any significant radiographic or histomorphometric improvement in non-critically sized defects in a rabbit cranial model when PRP was added to enhance bone formation. However, at 1, 2, and 4 months, a histomorphometric tendency for increased bone formation was observed when bone and bone/PRP were utilized.

In addition to the effect of PRP on bone formation in calvarial defects, other mechanisms involved in this process could explain the results obtained in the present study. Several studies of cranial suture biology have supported a role for the dura mater in the regulation of calvarial osteogenesis (30,47). Petrie et al. (48) showed that the dura mater has the ability to transform into cells characteristic of the two surrounding tissue types (bone and nerve) due to its neural crest origin, characterizing it as a multipotent cell type.

Gagan et al. (49) reported that cranial sutures are composed of a centrally-located group of proliferating osteoprogenitor cells, surrounded peripherally by the same cells undergoing osteogenic differentiation and programmed cell death. In addition, according to these authors, interactions of the dura mater with the brain and skull are very dynamic; mechanical and biochemical reciprocity has been observed in which the cells of the dura mater can influence cell migration and differentiation in several regions of the embryonic and infant brain and skull. These authors also suggested that the cells involved in the healing of a defect are derived from those located closest, and that differences in various populations of adult stem cells may reflect reparative capacity that is dependent on the organ involved and the tissue source of the stem cells. Due to their close proximity to both the bone and brain, it is possible that dura mater cells could be effective for treatment of cranial bone and neural deficits *in vivo*.

As PRP is an autologous source of concentrated growth factors, including TGF β , which affects bone formation as well as regulating and stimulating other growth factors, including FGF-2 (50,51), it can be suggested that this growth factor may have mediated the effects observed in the present study. Gosain et al. (52) showed that these specific growth factors (TGF β and FGF-2) have important roles in cranial suture development in the embryogenic and postnatal phases. Mehrara et al. (47) suggested that basic fibroblast growth factor (FGF-2) and transforming growth factor-beta1 (TGF- β 1) have osteogenic actions that are involved in regulating bone formation in calvarial bone defects. The present study demonstrated increased immunohistochemical staining

for FGF-2 and TGF- β 1 within calvarial osteoblasts with increasing age, especially in cells that were in contact with the developing dura mater. Based on the application of these modern principles to tissue regeneration and wound healing, a recent study of 10 patients with advanced frontal sinus disease treated with PRP and PPP and a bioactive scaffold demonstrated a favorable outcome within a follow-up period of 6-10 years, with no complications or sequelae (5).

The addition of BG has been used to achieve bone regeneration, based on the osteoconductive properties of this material (53). BG has become an alternative to autologous and allogeneic bone grafts, and has been employed in various clinical and animal studies (4,14,18-23,26). As shown by some previous authors (32,53), in the present study, no foreign body reaction was observed in the histological specimens, which even showed intense activity and areas of new bone formation, especially at the edge of the defects. These results imply that BG is biocompatible with the surrounding tissues.

A recently published systematic review evaluating the efficacy of PRP, combined with various therapeutic bioactive agents/procedures for the treatment of periodontal intraosseous defects found diverse outcomes (54). More than 6,000 potentially relevant titles and abstracts were examined in the research; however only 20 full-text articles were thoroughly evaluated. The authors concluded that in view of the limited and heterogeneous data available, additional research on the efficacy of these combinations is needed.

Although the present study radiographically demonstrated significantly higher bone density in the groups treated with PRP (as also reported by Marx et al. (6)) when it was used alone or combined with bone grafts, radiographic evaluation of bone repair should be done with caution. Pryor et al. (55) showed that radiographs had low accuracy for assessment of bone healing, for example in critical-size defects in rats. The authors considered that radiography does not reflect the actual stage of healing, and may underestimate the final process as well as overestimating healing at the intermediate stage. They also recommended that, before clinical application, this treatment method should be confirmed using histological analysis in animal models. However, it is important to point out that, in clinical situations, histological examinations are rarely possible and that radiographs can be of help in assessing bone formation. The histological outcomes in the present study indicated that bone formation occurred in all groups; however, the PRP groups demonstrated central focal areas of vital new bone formation, with or without BG. In both

groups treated with BG, macrophages were observed surrounding these particles.

With the evolution of animal and clinical studies, new knowledge and concepts have led to the addition, substitution, and alteration of therapies and clinical protocols. According to Simonpieri et al. (10) there was a transitional period in periodontology and oral maxillofacial surgery concerning the use of PRP and platelet-rich fibrin (PRF). The literature on this topic is still contradictory and published data showing actual benefits of PRP and BG for bone regeneration are limited. However, the use of platelet growth factors in association with cell or molecular therapies should be explored further, since these options may improve the process of periodontal or bone regeneration.

On the basis of the present results, it can be concluded that PRP improves bone repair in calvarial defects and that BG, alone or in combination with PRP, is ineffective for bone healing.

Acknowledgments

The authors thank the University Center of Maceió for structural support. No external funding was available for this study.

References

1. Camargo PM, Lekovic V, Weinlaender M, Vasilic N, Madzarevic M, Kenney EB (2002) Platelet-rich plasma and bovine porous bone mineral combined with guided tissue regeneration in the treatment of intrabony defects in humans. *J Periodontol Res* 37, 300-306.
2. Lekovic V, Camargo PM, Weinlaender M, Vasilic N, Aleksic Z, Kenney EB (2003) Effectiveness of a combination of platelet-rich plasma, bovine porous bone mineral and guided tissue regeneration in the treatment of mandibular grade II molar furcations in humans. *J Clin Periodontol* 30, 746-751.
3. Wiltfang J, Kloss FR, Kessler P, Nkenke E, Schultze-Mosgau S, Zimmermann R et al. (2004) Effects of platelet-rich plasma on bone healing in combination with autogenous bone and bone substitutes in critical-size defects. An animal experiment. *Clin Oral Implants Res* 15, 187-193.
4. Demir B, Sengün D, Berberoğlu A (2007) Clinical evaluation of platelet-rich plasma and bioactive glass in the treatment of intra-bony defects. *J Clin Periodontol* 34, 709-715.
5. Mendonça-Caridad J, Lopez PJ, Fayos FV, Miery G (2013) A novel approach to human cranial tissue regeneration and frontal sinus obliteration with an autogenous platelet-rich/fibrin-rich composite matrix: 10 patients with a 6-10 year follow-up. *J Tissue Eng Regen Med* 7, 491-500.
6. Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR (1998) Platelet-rich plasma: growth factor enhancement for bone grafts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 85, 638-646.
7. Garg AK (2000) The use of platelet-rich plasma to enhance

- the success of bone grafts around dental implants. *Dent Implantol Update* 11, 17-21.
8. Whitman DH, Berry RL, Green DM (1997) Platelet gel: an autologous alternative to fibrin glue with applications in oral and maxillofacial surgery. *J Oral Maxillofac Surg* 55, 1294-1299.
 9. Del Corso M, Vervelle A, Simonpieri A, Jimbo R, Inchingolo F, Sammartino G et al. (2012) Current knowledge and perspectives for the use of platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) in oral and maxillofacial surgery. Part 1: periodontal and dentoalveolar surgery. *Curr Pharm Biotechnol* 13, 1207-1230.
 10. Simonpieri A, Del Corso M, Vervelle A, Jimbo R, Inchingolo F, Sammartino G et al. (2012) Current knowledge and perspectives for the use of platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) in oral and maxillofacial surgery. Part 2: bone graft, implant and reconstructive surgery. *Curr Pharm Biotechnol* 13, 1231-1256.
 11. de Vasconcelos Gurgel BC, Gonçalves PF, Pimentel SP, Ambrosano GMB, Nociti Júnior FH, Sallum EA et al. (2007) Platelet-rich plasma may not provide any additional effect when associated with guided bone regeneration around dental implants in dogs. *Clin Oral Implants Res* 18, 649-654.
 12. Torres J, Tresguerres I, Tamimi F, Clemente C, Niembro E, Blanco L (2007) Influence of platelet-rich plasma on bone regeneration: a histomorphometric study in rabbit calvaria. *Int J Oral Maxillofac Implants* 22, 563-568.
 13. Casati MZ, de Vasconcelos Gurgel BC, Gonçalves PF, Pimentel SP, Nogueira-Filho GR, Nociti FH Jr et al. (2007) Platelet-rich plasma does not improve bone regeneration around peri-implant bone defects – a pilot study in dogs. *Int J Oral Maxillofac Surg* 36, 132-136.
 14. Brogini N, Hofstetter W, Hunziker E, Bosshardt DD, Bornstein MM, Seto I et al. (2011) The influence of PRP on early bone formation in membrane protected defects. A histological and histomorphometric study in the rabbit calvaria. *Clin Implant Dent Relat Res* 13, 1-12.
 15. Aghaloo TL, Moy PK, Freymiller EG (2002) Investigation of platelet-rich plasma in rabbit cranial defects: a pilot study. *J Oral Maxillofac Surg* 60, 1176-1181.
 16. Yazawa M, Ogata H, Kimura A, Nakajima T, Mori T, Watanabe N (2004) Basic studies on the bone formation ability by platelet rich plasma in rabbits. *J Craniofac Surg* 15, 439-446.
 17. Torres J, Tamimi F, Tresguerres IF, Alkhraisat MH, Khraisat A, Blanco L et al. (2010) Effect of combining platelet-rich plasma with anorganic bovine bone on vertical bone regeneration: early healing assessment in rabbit calvariae. *Int J Oral Maxillofac Implants* 25, 123-129.
 18. Simman R, Hoffmann A, Bohinc RJ, Peterson WC, Russ AJ (2008) Role of platelet-rich plasma in acceleration of bone fracture healing. *Ann Plast Surg* 61, 337-344.
 19. Nagata MJ, Melo LG, Messori MR, Bomfim SR, Fucini SE, Garcia VG et al. (2009) Effect of platelet-rich plasma on bone healing of autogenous bone grafts in critical-size defects. *J Clin Periodontol* 36, 775-783.
 20. Nagata M, Messori M, Okamoto R, Campos N, Pola N, Esper L et al. (2009) Influence of the proportion of particulate autogenous bone graft/platelet-rich plasma on bone healing in critical-size defects: an immunohistochemical analysis in rat calvaria. *Bone* 45, 339-345.
 21. Markou N, Pepelassi E, Vavouraki H, Stamatakis HC, Nikolopoulos G, Vrotsos I et al. (2009) Treatment of periodontal endosseous defects with platelet-rich plasma alone or in combination with demineralized freeze-dried bone allograft: a comparative clinical trial. *J Periodontol* 80, 1911-1919.
 22. Pradeep AR, Shetty SK, Garg G, Pai S (2009) Clinical effectiveness of autologous platelet-rich plasma and Peptide-enhanced bone graft in the treatment of intrabony defects. *J Periodontol* 80, 62-71.
 23. Mariano R, Messori M, de Moraes A, Nagata M, Furlaneto F, Avelino C et al. (2010) Bone healing in critical-size defects treated with platelet-rich plasma: a histologic and histometric study in the calvaria of diabetic rat. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 109, 72-78.
 24. Danesh-Meyer MJ, Filstein MR, Shanaman R (2001) Histological evaluation of sinus augmentation using platelet rich plasma (PRP): a case series. *J Int Acad Periodontol* 3, 48-56.
 25. Piemontese M, Aspriello SD, Rubini C, Ferrante L, Procaccini M (2008) Treatment of periodontal intrabony defects with demineralized freeze-dried bone allograft in combination with platelet-rich plasma: a comparative clinical trial. *J Periodontol* 79, 802-810.
 26. Camargo PM, Lekovic V, Weinlaender M, Divnic-Resnik T, Pavlovic M, Kenney EB (2009) A surgical reentry study on the influence of platelet-rich plasma in enhancing the regenerative effects of bovine porous bone mineral and guided tissue regeneration in the treatment of intrabony defects in humans. *J Periodontol* 80, 915-923.
 27. Oliveira-Filho MA, Nassif PA, Malafaia O, Ribas Filho JM, Ribas CA, Camacho AC et al. (2010) Effects of a highly concentrated platelet-rich plasma on the bone repair using non-critical defects in the calvaria of rabbits. *Acta Cir Bras* 25, 28-33.
 28. Oktay EO, Demiralp B, Demiralp B, Senel S, Cevdet Akman A, Eratalay K et al. (2010) Effects of platelet-rich plasma and chitosan combination on bone regeneration in experimental rabbit cranial defects. *J Oral Implantol* 36, 175-184.
 29. Aghaloo TL, Moy PK, Freymiller EG (2005) Evaluation of platelet-rich plasma in combination with freeze-dried bone in the rabbit cranium. A pilot study. *Clin Oral Implants Res* 16, 250-257.
 30. Plachokova AS, van den Dolder J, Stoelinga PJ, Jansen JA (2006) The bone regenerative effect of platelet-rich plasma in combination with an osteoconductive material in rat cranial defects. *Clin Oral Implants Res* 17, 305-311.
 31. Betoni-Junior W, Dechichi P, Esteves JC, Zanetta-Barbosa D, Magalhães AE (2013) Evaluation of the bone healing process utilizing platelet-rich plasma activated by thrombin

- and calcium chloride: a histologic study in rabbit calvaria. *J Oral Implantol* 39, 14-21.
32. Carvalho MD, Suaid FF, Santamaria MP, Casati MZ, Nociti FH Jr, Sallum AW et al. (2011) Platelet-rich plasma plus bioactive glass in the treatment of intra-bony defects: a study in dogs. *J Appl Oral Sci* 19, 82-89.
 33. Klongnoi B, Rupprecht S, Kessler P, Thorwarth M, Wiltfang J, Schlegel KA (2006) Influence of platelet-rich plasma on a bioglass and autogenous bone in sinus augmentation. An explorative study. *Clin Oral Implants Res* 17, 312-320.
 34. Keles GC, Cetinkaya BO, Albayrak D, Koprulu H, Acikgoz G (2006) Comparison of platelet pellet and bioactive glass in periodontal regenerative therapy. *Acta Odontol Scand* 64, 327-333.
 35. Trindade-Suedam IK, de Moraes JAND, Faeda RS, Leite FRM, Tosoni GM, Neto CB et al. (2010) Bioglass associated with leukocyte-poor platelet-rich plasma in the rabbit maxillary sinus: histomorphometric, densitometric, and fractal analysis. *J Oral Implantol* 36, 333-343.
 36. Jung RE, Schmoekel HG, Zwahlen R, Kokovic V, Hammerle CH, Weber FE (2005) Platelet-rich plasma and fibrin as delivery systems for recombinant human bone morphogenetic protein-2. *Clin Oral Implants Res* 16, 676-682.
 37. Aukhil I (2000) Biology of wound healing. *Periodontol* 2000 22, 44-50.
 38. Pietrzak WS, Eppley BL (2005) Platelet rich plasma: biology and new technology. *J Craniofac Surg* 16, 1043-1054.
 39. Kawase T, Okuda K, Saito Y, Amizuka N, Suzuki H, Yoshie H (2005) Platelet-rich plasma provides nucleus for mineralization in cultures of partially differentiated periodontal ligament cells. *In Vitro Cell Dev Biol Anim* 41, 171-176.
 40. Choukroun J, Diss A, Simonpieri A, Girard MO, Schoeffler C, Dohan SL et al. (2006) Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part V: histologic evaluations of PRF effects on bone allograft maturation in sinus lift. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 101, 299-303.
 41. Kanno T, Takahashi T, Tsujisawa T, Ariyoshi W, Nishihara T (2005) Platelet-rich plasma enhances human osteoblast-like cell proliferation and differentiation. *J Oral Maxillofac Surg* 63, 362-369.
 42. Banerjee C, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS et al. (1997) Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. *J Cell Biochem* 66, 1-8.
 43. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) *Osf2/Cbfa1*: a transcriptional activator of osteoblast differentiation. *Cell* 89, 747-754.
 44. Mooren RECM, Merkx MAW, Bronkhorst EM, Jansen JA, Stoelinga PJW (2007) The effect of platelet-rich plasma on early and late bone healing: an experimental study in goats. *Int J Oral Maxillofac Surg* 36, 626-631.
 45. Mooren RE, Dankers AC, Merkx MA, Bronkhorst EM, Jansen JA, Stoelinga PJ (2010) The effect of platelet-rich plasma on early and late bone healing using a mixture of particulate autogenous cancellous bone and Bio-Oss: an experimental study in goats. *Int J Oral Maxillofac Surg* 39, 371-378.
 46. Schmitz JP, Hollinger JO (1986) The critical size defect as an experimental model for craniomandibulofacial nonunions. *Clin Orthop Relat Res* 205, 299-308.
 47. Mehrara BJ, Most D, Chang J, Bresnick S, Turk A, Schendel SA et al. (1999) Basic fibroblast growth factor and transforming growth factor β -1 expression in the developing dura mater correlates with calvarial bone formation. *Plast Reconstr Surg* 104, 435-444.
 48. Petrie C, Tholpady S, Ogle R, Botchwey E (2008) Proliferative capacity and osteogenic potential of novel dura mater stem cells on poly-lactic-co-glycolic-acid. *J Biomed Mater Res A* 85, 61-71.
 49. Gagan JR, Tholpady SS, Ogle RC (2007) Cellular dynamics and tissue interactions of the dura mater during head development. *Birth Defects Res C Embryo Today* 81, 297-304.
 50. Garcia-Molina JA, Vallespi-Miró G, Greco-Machado Y, Perez-Tomas R, Ustrell-Torrent JM, Carvalho-Lobato P et al. (2004) The role of fibroblast growth factor (FGF) and type beta transforming growth factor (TGF- β 1- β 2- β 3) during rat craniofacial development. *Bull Group Int Rech Sci Stomatol Odontol* 45, 66-78.
 51. Shirakihara T, Horiguchi K, Miyazawa K, Ehata S, Shibata T, Morita I et al. (2011) TGF- β regulates isoform switching of FGF receptors and epithelial-mesenchymal transition. *EMBO J* 30, 783-795.
 52. Gosain AK, Recinos RF, Agresti M, Khanna A (2004) TGF- β 1, FGF-2, and receptor mRNA expression in suture mesenchyme and dura versus underlying brain in fusing and nonfusing mouse cranial sutures. *Plast Reconstr Surg* 113, 1675-1684.
 53. Vogel M, Voigt C, Gross UM, Müller-Mai CM (2001) In vivo comparison of bioactive glass particles in rabbits. *Biomaterials* 22, 357-362.
 54. Kotsovilis S, Markou N, Pepelassi E, Nikolidakis D (2010) The adjunctive use of platelet-rich plasma in the therapy of periodontal intraosseous defects: a systematic review. *J Periodontol Res* 45, 428-443.
 55. Pryor ME, Susin C, Wikesjö UM (2006) Validity of radiographic evaluations of bone formation in a rat calvaria osteotomy defect model. *J Clin Periodontol* 33, 455-460.